The effect of pre-treatment of protein ingredients for infant formula on their *in vitro* gastro-intestinal behaviour

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Abstract

Three milk products, skim milk powder (SMP), demineralised whey powder (DWP) and a whey dominant infant formula (60/40IF) and their corresponding partially hydrolysed products (SMPhyd, DWPhyd and 60/40hyd, respectively) were subjected to static infant *in vitro* gastro-intestinal (GI) digestion and their digesta were subsequently analysed for protein breakdown. The pre-hydrolysis of proteins provided a head-start in the gastric digestion process compared to the intact proteins, resulting in a higher proportion of small peptides (<1 kDa), a higher degree of hydrolysis and lower observable protein coagulation or curd formation in the gastric phase of the casein dominant systems in particular, which may lead to an earlier onset of gastric emptying *in vivo*. Little or no differences were detected during the intestinal phase. Hence pre-hydrolysis of proteins may be used as a strategy to lower gastric transit times which may ease the gastric digestion of infant formulations.

1. Introduction

Human milk is considered the nutritional gold standard for the newborn as it is tailored for the needs of the infant. In cases where the infant is not breast-feed it is important to provide infant formulas (IF) of the highest quality for adequate growth and development and the composition of these foods are strictly regulated (Codex-Alimentarius, 1987). Delivery of quality proteins that match the amino acid profile of human milk is to date mostly ensured by the formulation of bovine protein fractions, namely skim milk combined with whey protein concentrate from whey, a high quality protein by-product of cheese or casein production. First stage IF is designed to be the sole food for infants, hence whey protein based formulas have come to dominate the market place owing to the high nutritional value of whey proteins as well as their greater similarity to human milk when compared to casein based IF. The protein ratio of caseins to whey proteins in IF at 40:60, is similar to human milk but different

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to bovine milk which has an 80:20 ratio. It is thought that the lower levels of casein in both first stage IF and breast milk in comparison to bovine milk allows for faster gastric transit due to the formation of a softer curd during the gastric phase, leading to faster and easier gastro-intestinal (GI) digestion (Thompkinson & Kharb, 2007). The protein content in IF has also been lowered to mimic more closely the total protein levels found in human milk and eliminate excess levels of amino acids thought to cause metabolic stress in the infant (Fomon, 1991; Raiha, 1994). Second stage IF or Follow up Formula (FUF) typically contains a greater proportion of casein than first stage and mimics the protein profile of late stage breast milk (Kunz & Lonnerdal, 1992). Skim milk is typically added to infant formula to provide the casein element of the formula with whey, often in the form of whey protein isolate or other enriched whey components added to provide the majority of the whey protein (Schuck, Blanchard, & Zhu, 2013)

In contrast to plant based materials the main protein constituents of milk i.e. casein and whey proteins are readily digested by the human GI system. It would appear that the caseins have a longer residence time in the gastric phase than the whey proteins and this can be related to the coagulation of the caseins at their isoelectric point in the stomach by the action of gastric HCl (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2020). The exact mechanism and degree to which this governs slower gastric emptying of casein relative to whey protein into the duodenum is not clear. However, it is known that casein empties from the stomach in the form of degraded peptides whereas the whey proteins and β-lactoglobulin (β-lg) in particular, enter the duodenum as intact protein (Mahé, et al., 1996). Processing of milk is also known to affect the gastric curd, which is formed upon acidification and digestion by pepsin. Raw and pasteurised milk was shown to form a harder more compressed curd during semi-dynamic (Mulet-Cabero, et al.,

2019) as well as dynamic (Ye, Cui, Dalgleish, & Singh, 2016; Ye, et al., 2019) *in vitro* digestion compared to that of heated UHT milk. This was explained by the denaturation of the whey proteins and their association with the caseins during heat treatment as well as a re-distribution of calcium within the casein micelle, which affects the proteolysis by pepsin and the curd formation.

There are two main types of *in vitro* methods, static and dynamic; in static methods the food is subjected to preset physiological digestion parameters set to emulate the conditions found in the oral, gastric and intestinal phase. Although more accurate, the dynamic digestion methods which simulate the gradual addition of digestive fluids as well as continous gastric emptying into the intestinal tract are relatively complex, expensive to run, often commercially operated hence not standardised, and commonly unavailable to the majority of food researchers. Hence most published digestion studies use simpler static methods. To overcome the shortcomings such as variations in physiological conditions i.e. enzyme activities, dilutions and pH, of the different static models present at the time an international group of experts agreed to a consensus model for adult static *in vitro* digestion (Brodkorb, et al., 2019; Minekus, et al., 2014) also known as the INFOGEST method. However, no such consensus exists for different population groups (Levi, et al., 2017). For simulating infant digestion, the most commonly used static infant *in vitro* method is by Dupont *et al.,* (2010) , which was further refined and aligned with some aspects of the INFOGEST method by Menard *et al*. (2018).

The objective of this study was to assess the effect of pre-digestion of proteins, i.e. partial hydrolyses of the proteins in skim milk (SM), Demineralised Whey Protein (DWP) and a 60/40 ratio whey protein/casein mix (60/40IF) representing a model first-stage IF, on their digestive behaviour *in vitro* and compare this to existing evidence *in vivo.*

2. Materials and methods

2.1 Materials.

Six powder samples containing varying amounts of casein and whey proteins were provided by Kerry Group (Naas, Co. Kildare, Ireland). One group consisted of un-hydrolysed Skim Milk Powder (SMP), De-mineralised Whey Powder (DWP) and a whey dominant infant formula blend containing 60/40 whey protein to casein ratio (60/40IF). The second group consisted of the same set of powders, which had been partially hydrolysed (Degree of hydrolysis (DH): DWPhyd, 12.6%; SMPhyd, 12.2%, 60/40hyd a mixture of DWPhyd and SMPhyd, figures provided by Kerry Group). Compositional analysis was carried out in-house by the Moorepark Technical Service (Table 1). The protein content was determined by the Kjeldahl method (Bradstreet, 1954; Kjeldahl, 1883) using a nitrogen conversion factor (NCF) of 6.38 (Jones, 1931). The fat content was determined by the Rose Gottlieb method (AOAC, Arlington, USA, 1980). The moisture and ash were determined by gravimetric oven from LECO (LECO Instruments, Stockport, United Kingdom).

Each of the samples was prepared to 2% (w/w) protein and rehydrated overnight at 4ºC before being stirred for 2 hours at ambient temperature prior to digestion. Three independent sets of protein solutions were prepared for the experiments (n=3). All salts for the Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared using analytical grade chemicals supplied by Sigma-Aldrich (Sigma Chemical Co. St. Louis, USA). Rabbit Gastric Extract (RGE) was supplied by Lipolytech (Marseille, France). Pancreatin (P-7545, SLBV6830) and bile extract (B8631, 031MO106V) and all other reagents were sourced from Sigma-Aldrich (Sigma Chemical Co. St. Louis, USA) unless stated otherwise.

2.2 Methods

2.2.1 In vitro digestion of liquid meals

The food samples which had been re-hydrated overnight on a 2% (w/w) protein basis were subjected to both gastric and intestinal digestion according to the scheme of Menard *et al. (2018)*. This is the most recent *in vitro* infant digestion model reflecting the latest physiological data available. For the gastric digestion the samples were collected at time zero, with no enzyme (G0), after 30 min (G30) and finally after 60 min (G60). The samples for intestinal digestion were taken after the gastric digestion step was completed for 1h and then underwent intestinal digestion for either, 15 min (I15), 30 min (I30) or 60 min (I60). The time points were chosen to reflect the importance of both the gastric and intestinal endpoints and to allow sufficient time for preparation of the digesta so as to minimise the amount of error in the replicates. SGF and SIF were prepared and stored at 4ºC and all enzyme solutions were prepared on the day of the trial and stored on ice. The pancreatin added achieved the trypsin activity of 16 U/mL of intestinal content and covered the required lipase activity of 90 U/mL of intestinal content and the RGE added achieved the pepsin activity of 268 U/mL of gastric contents quoted by Menard *et al.* (2018). Porcine bile was added to give a final level of 3.1 mM of bile salts and calcium chloride was added separately to give a final intestinal concentration of 3 mM. The gastric phase was at pH 5.3 and the intestinal phase was at pH 6.6, which is based on available physiological data. For further justification of the infant digestion parameters, see Menard et al. (2018). All of the solutions were kept at 37ºC prior to digestion. Sample preparation of the gastric samples G0, G30 and G60 involved the adjustment of the pH to 7.0, followed by snap-freezing in liquid nitrogen. The digestion of

130 the intestinal samples was completed by adding Pefabloc® inhibitor to the samples before
131 snap-freezing in liquid nitrogen.

132 *2.2.2 Molecular weight distribution*

133 Size Exclusion Chromatography-High Performance Liquid Chromatography (SEC-HPLC)
134 was carried out to estimate the molecular weight distribution of proteins and digesta, using a
135 TSK G2000 SW_{xl} column (600 × 7.5 mm; Tosoh Bioscience GmbH, Stuttgart, Germany), on
136 a Waters 2695 HPLC with UV/ Visible detector and EMPOWER® software. Separation was
137 achieved by isocratic elution using 0.1% TFA in 30% acetonitrile. 10µl of 0.25% protein
138 solutions were filtered through a 0.45 µm PES filter prior to injection onto the column. A
139 series of molecular weight standards (GE Healthcare, Chicago, IL, USA), were ran on the
140 SEC to create a calibration curve including bovine serum albumin, carbonic anhydrase, β-lg,
141 α-lac, aprotinin, insulin chain b, bacitracin, histidine-tyrosine-leucine, phenylalanine and
142 glycine with molecular weights of 67000, 29000, 18400, 14400, 6500, 3496, 1400, 294, 165
143 and 75 Da, respectively. Molecular weight intervals were determined according to Gaspard *et*
144 *al.*(2019).

145

146 *2.2.3 Estimation of protein digestion*

147 The digested protein/peptide content in the *in vitro* samples was determined from the soluble
148 fraction of a 50:50 v/v mixture of sample and 24% (w/v) trichloroacetic acid (TCA) after
149 centrifugation at 3,000× g for 30 min using the Kjeldahl method using a NCF of 6.38. The
150 percentage of protein digestion or digestibility was then calculated according to the scheme
151 of Rudloff *et al.*(1992) by comparison with the estimated protein in the original digest minus
152 the amount found in the blank sample, which contained digestive enzyme but no food.

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155 *2.2.4 Protein characterisation*

156 Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) of the protein
157 products and the *in vitro* digests was carried out using a 4-12% Bis-Tris polyacrylamide gel
158 (Invitrogen, CA, USA). Samples were prepared under reducing conditions using NUPAGE®
159 sample agent containing ditritheriol (DTT). Samples were heated to 85ºC for 2 min to ensure
160 unfolding of the proteins. An unstained molecular weight ladder from Invitrogen (Invitrogen,
161 CA, USA) was also run in two lanes to determine the size of the proteins. The samples were
162 stained in instant blue stain (Expedeon, Cambs., UK). The gels were de-stained in MilliQ®
163 water before image analysis using an Epson Scanner (Epson-Telford Ltd., Telford, UK).

164

165 *2.2.5 Protein hydrolysis*

166 The levels of the free amine groups in the digests were determined by o-phthaldialdehyde
167 (OPA) 96-well micro-assay using the method of Spellman *et al.*(2003). A calibration curve
168 was prepared as described by Mulet-Cabero *et al.* (2019) from a set of L-leucine standard
169 solutions between 0-10 mM. 10µL of either the standard or sample was then mixed with 200
170 µL of OPA for 15 min and the resultant absorbance was measured in a microplate reader
171 (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 340nm.

172

173 *2.2.6 Statistical analysis*

All statistical analysis was carried out using Minitab software (Minitab Inc.,PA, USA) with comparison of the means using Tukey's model (n=3).

3. Results

3.1 Visual assessment of digests

SMP, DWP, 60/40IF and their respective hydrolysed products were exposed to *in vitro* gastric and intestinal digestion based on the most recent static infant method (Ménard, et al., 2018). The hydrolysed samples DWPhyd and 60/40hyd were largely translucent and remained so during both the gastric and intestinal phase. The 60/40IF sample was turbid and white because of the intact casein micelles present in the dispersion but the 60/40hyd sample was more translucent as the casein present had been pre-hydrolysed. The control DWP and 60/40IF whey protein dominant formulas became largely translucent at the end of the intestinal phase owing to the action of the intestinal enzymes. Significant differences were observed during the gastric digestion of SMP compared to SMPhyd. The images in Fig. 1 A show the SMP dispersion before digestion (in standard laboratory Petri dishes), which has a milk-white colour due to the presence of 80% casein in micellar form. In contrast to this, the hydrolysed form of SMP is more translucent (Fig. 1 D) due to the pre-digestion of caseins, which destabilises the casein micelle and reduces the turbidity. The SMP sample was observed to curdle after 5 min of gastric digestion due to protein aggregation induced by the change in pH to 5.3 and the action of pepsin. However, no curdling was observed in the SMPhyd sample throughout the gastric digestion (Fig. 1 E and F). After 60 min of gastric digestion the SMP sample contained a greater amount of visibly aggregated material (Fig. 1 C) compared to the equivalent hydrolysed sample (Fig. 1 F). These differences between SMP

197 and SMPhyd disappeared during intestinal digestion due to the rapid action of pancreatic
198 enzymes, and both digested samples appeared translucent (not shown).

199

200

201 **3.2 Protein profile**

202 Digested protein products were analysed by SEC-HPLC, whereby water-soluble proteins and
203 peptides are separated by size and grouped into size intervals, see Fig. 2. Results can be
204 correlated to the extent of proteolysis into small and medium peptides. From the SEC data a
205 number of differences were observed between the control and hydrolysed samples for the
206 gastric phase. When the molecular weight profiles (Fig. 2 A, B and C) for the samples before
207 digestion (G0), were compared to the profiles from the gastric samples after 30 min (G30)
208 and 60 min (G60) of digestion, clear differences were observed. In the control SMP samples
209 (Fig. 2 A), the proportion of the two largest molecular weight materials (>30 kDa and 20-30
210 kDa), which are associated to intact proteins and aggregates, decreased significantly ($p<0.05$)
211 as gastric digestion progressed. Conversely, the proportion of the smaller molecular weight
212 (1-5 kDa and <1 kDa) material increased significantly ($p<0.05$) during gastric digestion. The
213 molecular weight profiles of SMPhyd were largely unaffected over the same time, except for
214 some significant ($p<0.05$) differences in the 5-10 kDa material. Overall, there was a
215 significantly ($p<0.05$) higher proportion of low molecular weight material (<1 kDa) in the
216 SMPhyd samples in comparison to its corresponding control SMP. The proportion of small
217 molecular weight material (1-5 kDa and <1 kDa) in both SMPhyd and SMP increased
218 significantly ($p<0.05$) during intestinal digestion in comparison to the gastric phase, mainly

219 due to the efficient action of the pancreatic proteases including trypsin and chymotrypsin
220 (Guo, Fox, Flynn, & Kindstedt, 1995; Tunick, et al., 2016).

221 The whey proteins in the control DWP samples (Fig. 2 B) largely resisted gastric proteolysis
222 and no changes were observed in their molecular weight profiles. In DWPhyd samples the
223 proportion of the largest material (>30 kDa) decreased significantly ($p<0.05$) during the
224 gastric phase. Comparing DWP and DWPhyd, there was significantly ($p<0.05$) more low
225 molecular weight material (<1.0 kDa) present in the pre-hydrolysed DWPhyd throughout the
226 gastric phase. There was a significantly ($p<0.05$) greater proportion of small (<1 kDa)
227 molecular weight material in both the hydrolysed and control DWP samples after intestinal
228 digestion (I15, I30 and I60) in comparison to the gastric samples (G30 and G60) as well as
229 G0.

230 A small but significant ($p<0.05$) decrease in the proportion of the high (>30 kDa) molecular
231 weight material in the 60/40hyd (Fig. 2 C) after both 30 and 60 minutes gastric digestion, was
232 observed when compared with the undigested sample (G0). There was no significant increase
233 or decrease in the proportion of the different molecular weight materials after 30 and 60 min
234 gastric digestion of the 60/40IF when compared to the sample prior to digestion (G0).

235 There was a significantly ($p<0.05$) greater proportion of small (<1 kDa) molecular weight
236 material in both 60/40IF and 60/40hyd after intestinal digestion (I15, I30 and I60) in
237 comparison to their respective gastric samples (G30, G60 and G0).

238 The percentage of <1 kDa molecular weight material in both the DWP and DWPhyd samples
239 for the gastric phase appeared to be lower than the SMP and SMPhyd samples (Fig. 2 B and
240 C). This is probably related to the higher percentage of casein present in the SMP, which is
241 preferentially hydrolysed by pepsin, whereas the major whey proteins β-lg and α-lactalbumin

(α-lac), are known to resist or delay, but not prohibit the proteolysis by pepsin, mainly due to the compact native globular structure of the whey proteins (de Oliveira, et al., 2016; de Oliveira, et al., 2017; Sanchón, et al., 2018; Sullivan, Mok, & Brodkorb, 2013).

3.3 Protein identification by SDS-PAGE

The proteolytic action of both the gastric and intestinal enzymes can be clearly seen in the SDS-PAGE gel electrograms (Fig. 3 A, B and C). There are three bands apparent between 20-30 kDa in the un-digested G0 sample for SMP, corresponding to the three major casein groups α, β and κ-Casein, and two bands for the whey proteins β-lg at ~18kDa α-lac at ~14kDa. After 30 minutes of gastric digestion (G30) there is a clear decrease of the casein bands in comparison with G0, with a further diminution in intact protein observed at G60. The whey proteins in the SMP remained largely intact throughout gastric digestion. No bands corresponding to either intact casein or whey proteins were observed in the SMPhyd (Fig. 3 A).

The SDS-PAGE of DWP (Fig 3 B) clearly show the bands corresponding to intact α-lac and β-lg, which remain unchanged during the gastric phase. For the DWPhyd samples faint bands of both whey proteins, which decrease in intensity during the gastric phase are visible. However, no whey proteins bands of DWP and DWPhyd are detected during the intestinal phase.

The SDS-PAGE electrograms of 60/40 IF (Fig. 3 C) displayed differences between the casein bands for G0, G30 and G60. G0 has three bands corresponding to α, β and κ-Caseins, whereas G30 and G60 have only fainter casein bands. The intensity of the bands corresponding to the whey proteins are not reduced upon gastric digestion. For the 60/40hyd there are no intact casein bands present and the faint whey protein bands are again reduced

during gastric digestion. No bands for casein or whey proteins appear in the intestinal digests except those of the digestive enzymes.

3.4 Estimation of protein digestion

The estimation of the protein digestion or percentage of digestibility was based on the quantification of TCA-soluble protein/peptides material (Calsamiglia & Stern, 1995; Rudloff & Lönnerdal, 1992), i.e. the TCA-soluble fraction in digested samples compared to the estimated protein in the original samples using the Kjeldahl method and a NCF of 6.38. The protein material coming from the enzyme extracts (pepsin and pancreatin) in blank experiments were deducted from quantified proteins in the digested samples. Only small peptides and amino acids remain soluble in 12% TCA (Yvon, Chabanet, & Pélissier, 1989) hence its use as an estimation of bioaccessible peptides and amino acids.

The hydrolysed and un-hydrolysed SMP, DWP and 60/40IF samples were prepared and analysed in triplicate (n=3). As expected, the hydrolysed products SMPhyd, DWPhyd and 60/40hyd (Table 2) have high proportions of TCA-soluble protein material i.e. 87, 49 and 64%, respectively. Un-hydrolysed SMP, DWP and 60/40IF also contain TCA-soluble protein material i.e. 2.4, 4.7 and 4.2%, respectively. The Kjeldahl nitrogen results include small amounts of free amino acids and non-protein nitrogen, which amount to approximately 1% in milk and SMP (Lindmark-Månsson, Fondén, & Pettersson, 2003; McDermott, et al., 2016). Due to the manufacture of whey proteins, this proportion is expected to be higher in both DWP and 60/40IF. However, the effect of the initial NPN and free amino acids on the protein content in the TCA-soluble fraction is reduced upon progression of proteolysis during GI digestion. The data in Table 2 show that the gastric sample after 60 min (G60) for the SMP has a greater digestibility at 8.73% than G0 at 2.37%. The same trend was observed for the both DWP and 60/40IF sample, with digesta at G60 having significantly higher ($p<0.05$)

289 TCA-soluble protein material in comparison to those at G30 and G0. When comparing the
290 same gastric time points of the ingredients together, significant differences were observed.
291 The SMPhyd samples had a significantly ($p<0.05$) higher digestibility at G30 and G60 (88.4
292 and 83.9%, respectively) than the same gastric digestion time points for the DWPhyd sample
293 (44.1 and 46.2%, respectively). Similar trends were observed for both DWP vs. DWPhyd and
294 60/40IF vs. 60/40hyd during gastric digestion. It was also noted that based on the TCA-
295 solubility, hydrolysed protein products were largely unaffected by gastric digestion, except
296 for some significant ($p<0.05$) difference for SMPhyd at G60.

297 When looking at the intestinal digestions at the I15, I30 and I60 time points (Table 2) there
298 did not appear to be significant differences in digestibility for the hydrolysed or non-
299 hydrolysed samples i.e. the large differences observed for the gastric phase were negated in
300 the intestinal digestion phase.

301

302 **3.5 Free amine determination by o-phthaldialdehyde assay (OPA)**

303 The OPA assay quantifies the relative amount of free amine groups liberated due to the
304 cleavage of peptide bonds, and is therefore suitable indicator for the extent of proteolysis.
305 OPA results are largely in line with all other results presented in this study. All gastric
306 samples (Fig. 4) both before (G0) and after 30 and 60 min (G30 and G60) digestion had
307 significantly ($p<0.05$) lower levels of free amine groups than the samples which had
308 undergone intestinal digestion (I15, I30 and I60). The gastric samples of SMP, DWP and
309 60/40IF contained significantly ($p<0.05$) lower amounts of free amine groups in comparison
310 to SMPhyd, DWPhyd and 60/40hyd due to pre-digestion of these samples.

There was a significantly lower amount ($p<0.05$) of free amine groups in G0 of both the SMP and SMPhyd than after both 30 and 60 min gastric digestion when the gastric time points were compared together. This trend was repeated for the DWP and 60/40IF gastric samples. The intestinal samples (I15, I30 and I60) were not significantly different from one another confirming the high degree of proteolysis during the intestinal phase compared to the gastric phase.

4. Discussion

The results of the study undertaken here show that pre-hydrolysing proteins appeared to aid the speed of gastric digestion of both the protein ingredients (SMPhyd, DWPhyd) as well as the resulting first stage infant protein formula (60/40hyd) when compared to their non-hydrolysed counterparts. This is simply due to the hydrolysed proteins having a head-start in proteolysis during gastric digestion. After intestinal digestion the differences in digestibility, based on the TCA-soluble Kjeldahl protein nitrogen and free amine group analysis, disappeared completely.

From the SEC data it could be seen that the hydrolysed SMP had a greater proportion of smaller molecular weight material (<1 kDa) and lower proportion of higher molecular weight material (20-30 and > 30 kDa) than the non-hydrolysed SMP, throughout the gastric phase. This was due to the cleavage of both the caseins and whey proteins by the proteolytic action of commercial enzymes, which had hydrolysed the proteins more effectively than the pepsin in the gastric phase. This is supported by the SDS-PAGE gels, which show a complete

hydrolysis of the casein and indeed whey protein bands in the SMPhyd samples and to a lesser extent the partial hydrolysis of the β-lg and α-lac in both the DWPhyd and 60/40hyd samples.

The intact β-lg and α-lac of SMP, DWP and 60/40IF (Fig. 3) are not degraded to proteolytic products by gastric digestion alone as seen in the SDS-PAGE. The finding that the whey proteins in particular are more resistant to the action of the gastric enzymes concurs with the earlier findings of infant *in vitro* (de Oliveira, et al., 2016) and adult *in vivo* (Sanchón, et al., 2018) studies.

It is interesting to note that the remaining intact whey proteins in DWPhyd are hydrolysed by pepsin due to the commonly used industrial practice of heat-inactivation of the commercial enzymes after hydrolysis, which causes irreversible denaturation and aggregation of the whey proteins β-lg and α-lac. Unfolding and in particular aggregation of whey proteins has been shown to increase enzyme accessibility, which can accelerate its degradation (O'Loughlin, Murray, Kelly, FitzGerald, & Brodkorb, 2012). The SDS-PAGE and SEC data for the intestinal digestion showed a complete degradation of the SMP, DWP and IMF samples irrespective of whether they had been pre-hydrolysed or not. This is not surprising as pancreatic enzymes, trypsin and chymotrypsin in particular, are strong proteolytic enzymes (Kim, et al., 2007).

Hydrolysed proteins in nutritional infant products are generally used for the purpose of avoiding or reducing the allergenicity of intact proteins (Alles, Scholtens, & Bindels, 2004; Zeiger, Heller, Mellon, O'Connor, & Hamburger, 1986) and the degradation of allergenic epitopes (Chobert, 2012). The current study presents evidence *in vitro* that the hydrolysis of protein products can reduce or prevent protein coagulation in the gastric phase, in particular for SMP but also 60/40IF. The coagulation observed in the static *in vitro* digestions is a result of

pepsin digestion at a constant pH of 5.3. Under physiological conditions, the pH is gradually lowered to pH 3-3.5 with increasing pepsin addition. This is likely to result in an even stronger protein coagulation due to the longer time frame of gelation, compared to the sudden drop in pH experienced in the static models. This effect could be simulated in dynamic or semi-dynamic models (de Oliveira, et al., 2015; Mulet-Cabero, Torcello-Gómez, et al., 2020; Mulet, et al., 2019; Ye, et al., 2019). The coagulation observed for the SMP sample at pH 5.3 was probably due to two factors namely, the partial proteolysis of the caseins by pepsin, which can retain activity up to pH 5.5 (Piper & Fenton, 1965) and a concomitant pH induced coagulation of the SMP as observed in previous studies (Lucey, Teo, Munro, & Singh, 1997; Lucey, Tamehana, Singh, & Munro, 1998). Huppertz & Lambers (2020) recently suggested that the micellar calcium phosphate content also influenced the gastric coagulation behaviour of infant formula *in vitro*. The gastric transit i.e. (i) feeding, (ii) gastric restructuring by pH, enzymatic hydrolysis and peristalsis and (iii) gastric emptying into the duodenum are key factors in the overall kinetics of protein digestion. Pepsin is thought to act on milk in a similar manner as chymosin, which hydrolyses the Phe_{105} – Met_{106} peptide bond of κ-casein during cheese manufacture releasing caseinomacropeptide (CMP) and causing the milk to coagulate (Hooydonk, Olieman, & Hagedoorn, 1984). The gastric coagulation of protein can be modulated by pre-treatments such as heating, by formulation (Mulet-Cabero, Rigby, et al., 2020) or by enzymatic hydrolysis of the proteins as demonstrated in this study. Differences in the physical properties of the coagulum have been shown to affect the kinetics of gastric emptying, appetite, satiety and feeling of fullness (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Mulet-Cabero, Rigby, et al., 2020); liquid gastric contents are emptied easier and faster.

The ease of digestion for infant formulations is thought to be important for the growing infant from the viewpoint of both abdominal discomfort as well as nutritional uptake and bio-

382 accessibility (Boirie, et al., 1997; Gan, Bornhorst, Henrick, & German, 2018). Clinical trials
383 with adults as well as studies using dynamic or semi-dynamic digestion methods seem to point
384 towards gastric restructuring as a cause for changes in overall digestive kinetics and gastric
385 emptying in particular. Only a small number of *in vivo* infant studies have correlated the use of
386 hydrolysed proteins with gastric emptying kinetics. Mihatsch et al. (2001) observed with
387 preterm infants (n = 15) that hydrolysed protein formula (75 % of the protein was smaller than
388 1,500 Da) resulted in a significantly ($p < 0.0022$) shorter total gastro-intestinal transit time (9.8
389 h) compared to standard preterm formula containing intact proteins (19 h). In a later study
390 Mihatsch et al. (2005) proposed that the pre-hydrolysis of casein reduces the opioid activity of
391 some peptides released from intact caseins during GI digestion with adult rats thereby
392 accelerating total GI transit compared to intact caseins. Another study using the adult rat model
393 (n = 8 to 15) showed that the gastric emptying time measured by x-ray was unaffected by the
394 pre-hydrolysis of caseins whereas the whey proteins emptied faster than both hydrolysed whey
395 proteins and caseins (Dalziel, Young, McKenzie, Haggarty, & Roy, 2017). An extensively
396 hydrolysed formula (88% of the protein smaller than 1,500 Da) showed a significantly ($p <$
397 0.05) faster gastric emptying time in healthy newborns (n = 20, measured by breath ^{13}C-
398 octanoic acid) compared to both intact protein formula and partially hydrolysed formula
399 (Staelens, et al., 2008); however no details on the extent of hydrolysis of the latter product
400 were provided in the paper. The authors concluded that IF with extensively hydrolysed proteins
401 may be better tolerated by infants with gastric emptying problems. An international working
402 group consensus on the recommendation of partially hydrolysed formula concluded that
403 "partially hydrolysed whey based formula is likely to result in faster gastric emptying than
404 formula based on intact protein, but the clinical relevance, for instance with respect to gastric
405 digestion, of this finding has not been demonstrated in term infants" (Vandenplas, et al., 2016).
406 Median gastric emptying time as measured by real-time ultrasonography in preterm infants

(triple-blind, controlled trial) was significantly ($p \leq 0.018$) faster using extensively hydrolysed formula compared to control formula (Baldassarre, et al., 2019). Other benefits of hydrolysed protein formula include the reduction in oesophageal acid exposure in preterm infants (gestational age $\leq$ 33 week, randomised crossover trial) with feeding intolerance and symptoms of gastro-oesophageal reflux (Corvaglia, Mariani, Aceti, Galletti, & Faldella, 2013). Hydrolysed whey proteins in combination with higher concentration sn-2 palmitic acid and prebiotic oligosaccharides resulted in a strong tendency (but no statistical significance) of softer stools in constipated infants (n = 35, randomised crossover trial). Hence, to date there is no clear consensus on the matter, even though some results point towards a shorter digestion time, which is generally associated with easier digestion. However, most clinical studies with hydrolysed protein formulas were conducted using relatively small numbers of infants compared to larger studies (Alarcon, Tressler, Mulvaney, Lam, & Comer, 2002) correlating protein re-formulation (e.g. caseins vs. whey proteins enriched formula) with changes in the gastric emptying, among others (n=6,999 in 17 countries). Many clinical studies also fall short of correlating the molecular or micro-structural changes with the mechanism and kinetics of GI digestion. More invasive techniques such as neonatal naso-gastric aspiration (de Oliveira, et al., 2017) in combination with *in vitro* digestion studies are necessary for any meaningful correlation to be drawn between the food, digestion and health, though such *in vivo* studies have clear ethical limitations as regards to risk vs. benefit and fewer and fewer studies are currently being conducted.

For the protein products presented in this study, it is reasonable to assume that hydrolysed proteins are emptied faster than products containing intact proteins. This might aid their ease of digestion, based on some of the in vivo studies mentioned above, and thus help to reduce some symptoms of discomfort. More sophisticated digestion models such as the consensus semi-

dynamic models (Mulet-Cabero, Egger, et al., 2020) can also be applied to study gastric coagulation behaviour more accurately (Mulet-Cabero, Torcello-Gómez, et al., 2020). Efforts within the research community, such as the INFOGEST network, are being made to agree on an acceptable consensus on digestion methods for population groups such as infants (Levi, et al., 2017).

5. Conclusions

Infant *in vitro* digestion of protein ingredients and model infant formula provide a good insight into the mechanism of protein digestion. The results of the study showed that pre-treatment such as partial hydrolysis of proteins can accelerate the gastric digestion of proteins in ingredients and model infant protein formulations compared to the equivalent non-hydrolysed, samples containing whole proteins. This is particularly true when comparing the protein and peptide pattern at the end of the gastric phase, where pre-treated proteins already exhibited a higher degree of proteolysis even prior to digestion. The final digestion product after intestinal digestion seemed largely unaffected by pre-treatment. From the SDS-PAGE data it also appeared that the gastric enzymes acted faster on α-lac than β-lg, which correlates well with available *in vivo* data. The information of this study could be used to help design formula, which would have lower GI transit times and help design easier to digest formula for infants where breastfeeding is not an option.

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Contributions:

The authors B.C. and A.B. designed the study, compiled all results and wrote the manuscript. B.C. carried out all experimental work including data and statistical analysis.

Conflict of interest

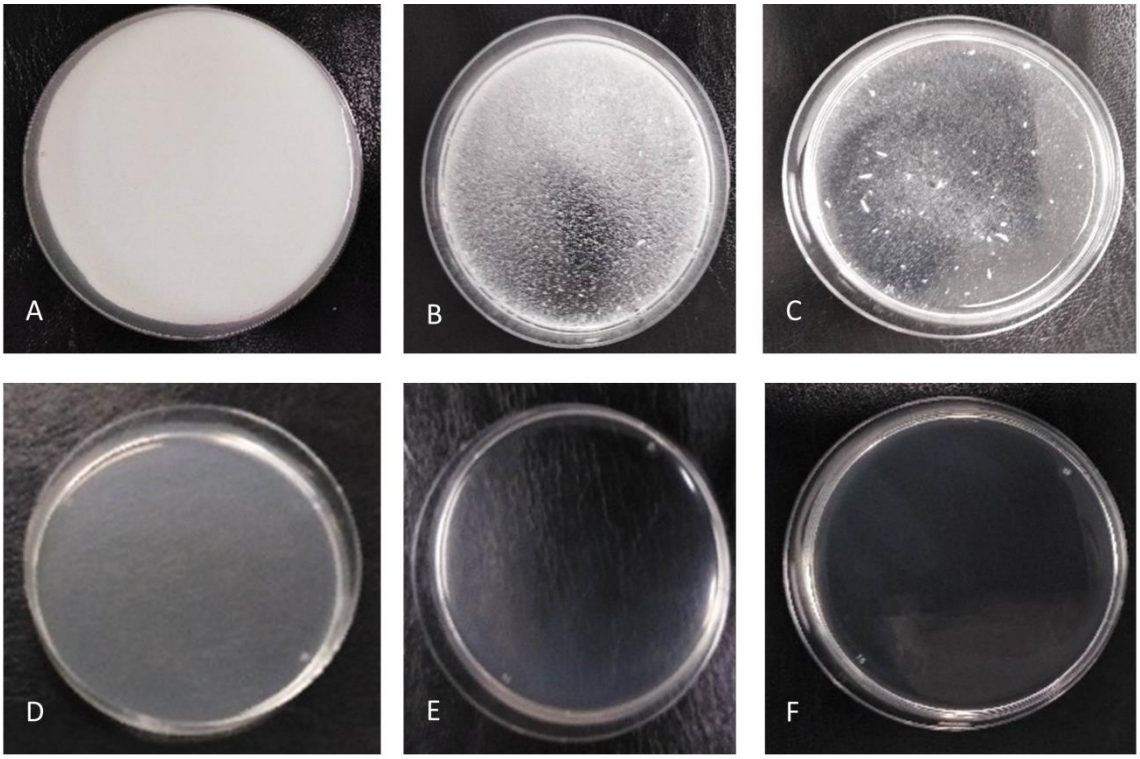
There is no conflict of interest. The study was financed by Kerry group under a contract agreement with Teagasc. The authors B.C. and A.B. did not financially benefit from this contract.

Table 1: Average compositional analysis of powders for trials ± standard deviation.

	Protein %	Fat %	Lactose %	Moisture %	Ash %
SMP	35.9 ± 0.09	0.71 ± 0.07	51.3 ± 0.03	4.40 ± 0.02	7.74 ± 0.01
SMPhyd	35.3 ± 0.09	0.76 ± 0.21	57.0 ± 0.28	2.55 ± 0.24	4.31 ± 0.58
DWP	12.2 ± 0.02	0.80 ± 0.02	84.3 ± 10.1	2.06 ± 0.03	0.65 ± 0.08
DWPhyd	12.9 ± 0.01	0.93 ± 0.13	82.7 ± 0.25	2.09 ± 0.02	1.35 ± 0.09
60/40IF	19.2 ± 0.21	0.99 ± 0.04	74.1 ± 0.17	2.21 ± 0.04	3.47 ± 0.01
60/40hyd	18.5 ± 0.23	0.83 ± 0.03	75.4 ± 0.16	2.80 ± 0.03	2.53 ± 0.05

Table 2: Protein content of the TCA-soluble fraction determined by the Kjeldahl method (NCF 6.38) before, during and after *in vitro* digestion: gastric time points G0, G30 and G60 and intestinal time points I15 and I60 for SMP, DWP and 60/40IF in comparison to corresponding time points for pre-hydrolysed samples SMPhyd, DWPhyd and 60/40hyd, respectively. Lower case letters denote significant differences across rows, while upper case letters denote significant differences between means down columns (n = 3).

	G0	G30	G60	I15	I30	I60
SMP	2.37±0.65$^{\mathbf{bD}}$	6.77±0.62$^{\mathbf{aD}}$	8.73±1.59$^{\mathbf{aD}}$	82.8±3.56	85.8±5.19	91.2±11.7
SMPhyd	86.7±2.75$^{\mathbf{aA}}$	88.4±4.74$^{\mathbf{aA}}$	83.9±8.53$^{\mathbf{bA}}$	96.9±2.91	95.6±6.55	92.2±2.69
DWP	4.66±1.01$^{\mathbf{bD}}$	6.42±0.48$^{\mathbf{bD}}$	9.04±0.90$^{\mathbf{aD}}$	87.2±0.36	89.9±7.15	93.2±5.12
DWPhyd	49.4±8.73$^{\mathbf{aC}}$	44.1±2.30$^{\mathbf{aC}}$	46.2±2.35$^{\mathbf{aC}}$	85.5±3.72	97.7±9.59	87.1±10.5
60/40IF	4.22±0.13$^{\mathbf{cD}}$	7.41±0.45$^{\mathbf{bD}}$	8.89±0.53$^{\mathbf{aD}}$	84.5±5.77	82.9±9.12	88.1±4.12
60/40hyd	64.3±2.26$^{\mathbf{aB}}$	63.6±3.04$^{\mathbf{aB}}$	66.5±5.40$^{\mathbf{aB}}$	83.3±2.34	88.0±6.69	82.2±9.46

667 

668 **Fig. 1:** Example images of digestion of SMP and SMPhyd using static *in vitro* digestion:
669 SMP reconstituted at 2% (w/w) protein before digestion (A), after 5 min gastric digestion (B)
670 and after 60 min gastric digestion (C); SMPhyd reconstituted at 2% (w/w) protein before
671 digestion (D), after 5 min gastric digestion (E) and after 60 min gastric digestion (F).

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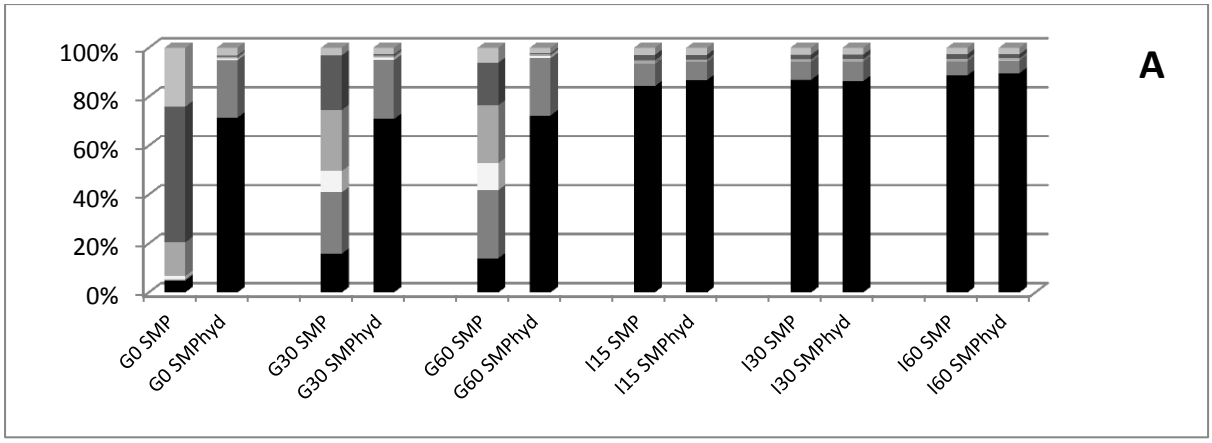

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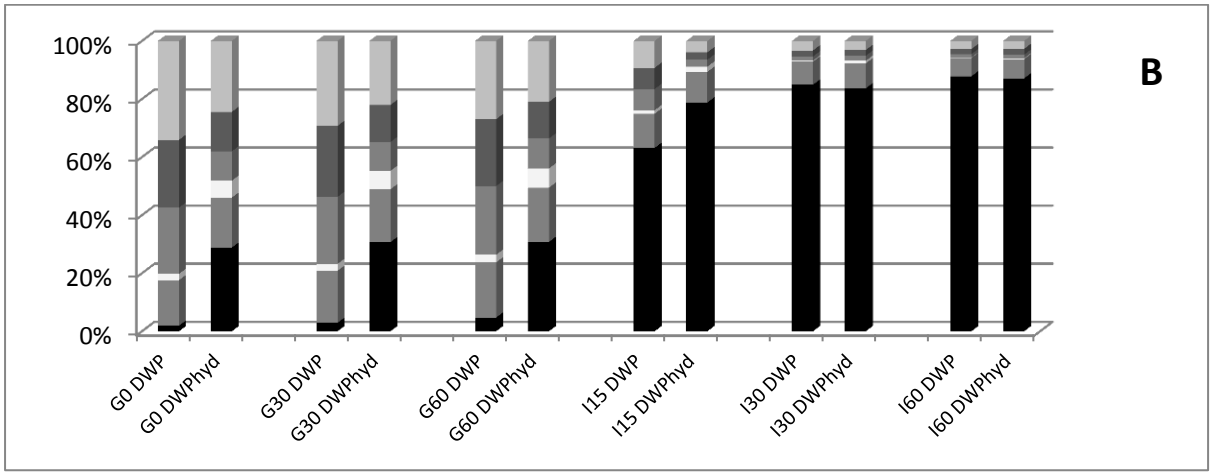

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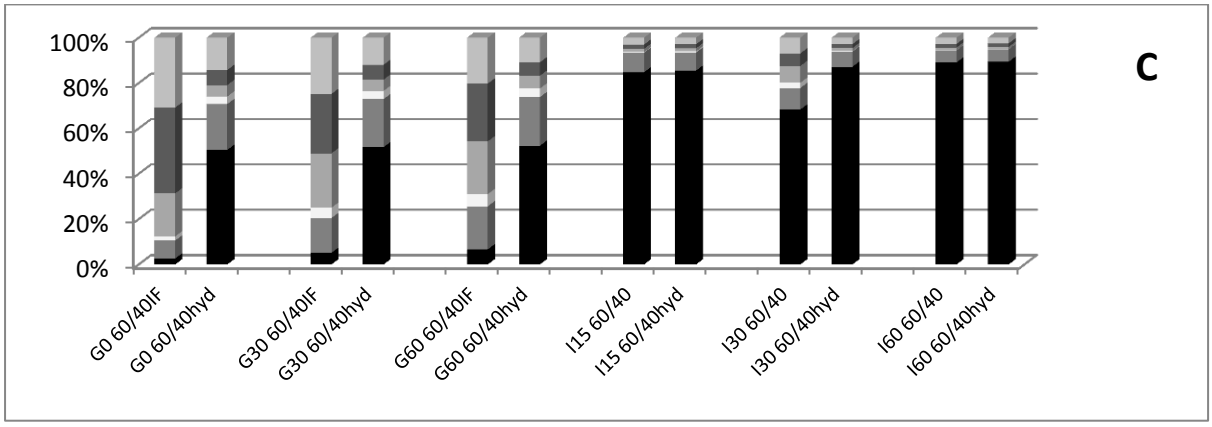

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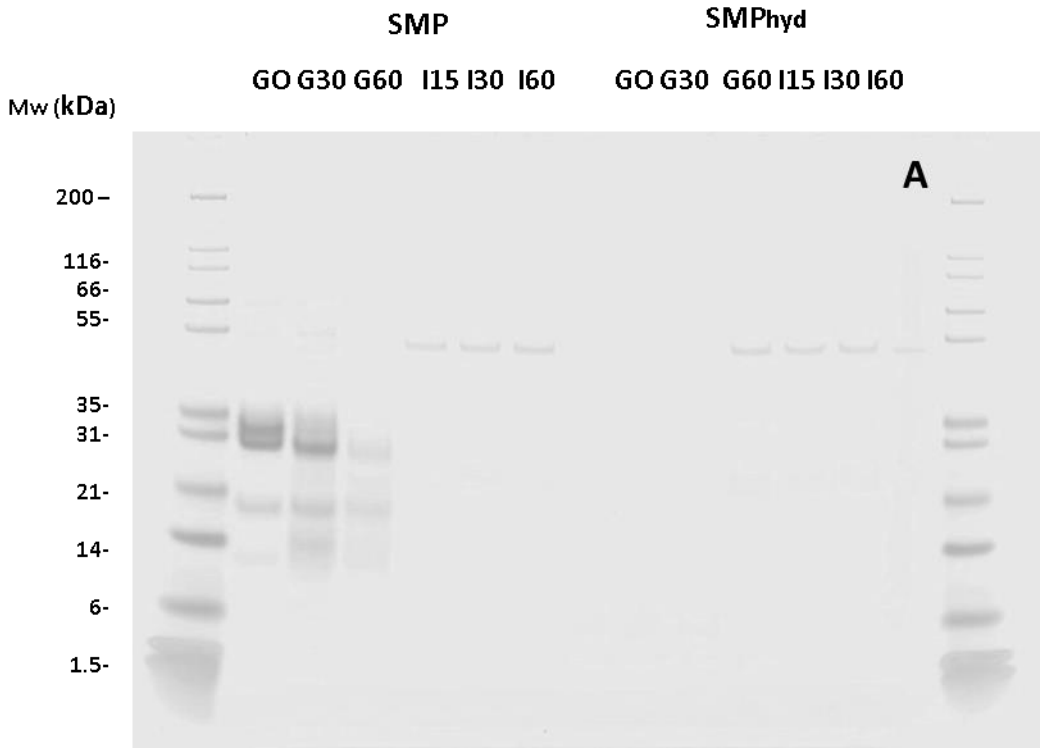
678 **Fig. 2:** Molecular weight distribution as determined by size exclusion HPLC of the soluble proteins
679 and peptides; <1 kDa (▬), 1-5 kDa (▬), 5-10 kDa (▬), 10-20 kDa(▬), 20-30kDa (▬), >30
680 kDa (▬) of digesta from *in vitro* static digestion of (A) skim milk (SMP) compared to hydrolysed
681 skim milk (SMPhyd), (B) demineralised whey (DWP) compared to hydrolysed demineralised whey
682 (DWPhyd) and (C) an infant formula protein blend with a 60/40 whey protein to casein ratio
683 (60/40IF) compared to a hydrolysed equivalent (60/40hyd).



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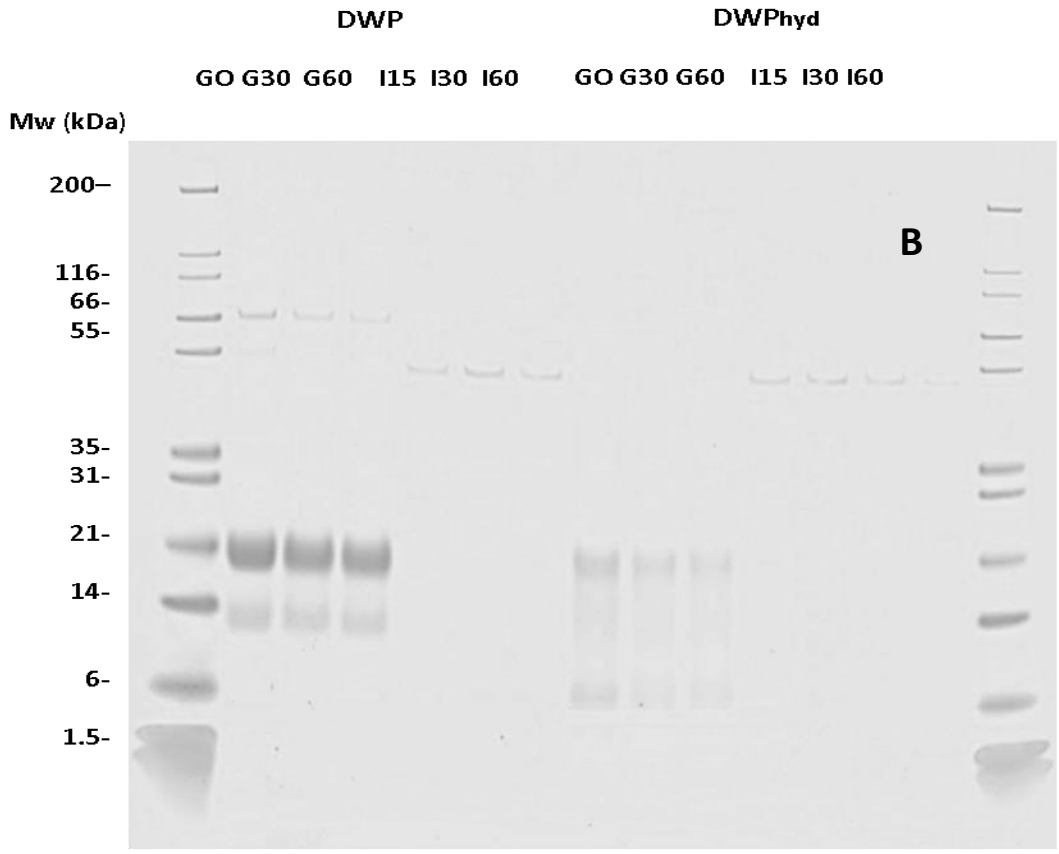

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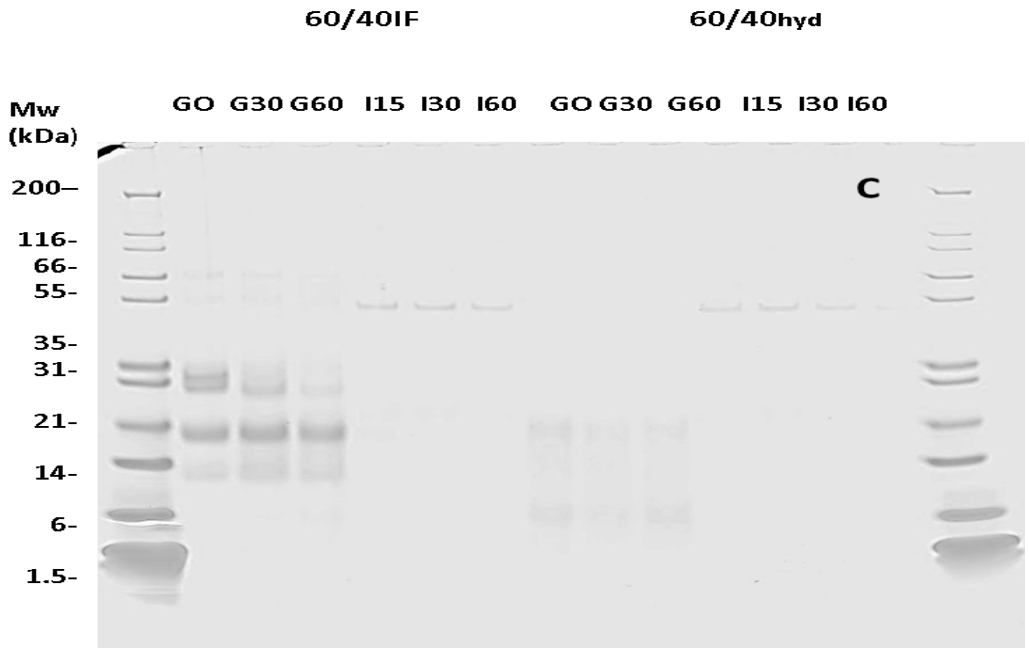

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693 **Fig. 3:** SDS-PAGE protein profiles of static digestion of (A) skim milk (SMP) compared to
694 hydrolysed skim milk (SMPhyd); (B) demineralised whey (DWP) compared to hydrolysed
695 demineralised whey (DWPhyd) and (C) an infant formula protein blend with a 60/40 whey
696 protein to casein ratio (60/40IF) compared to the hydrolysed equivalent (60/40hyd).

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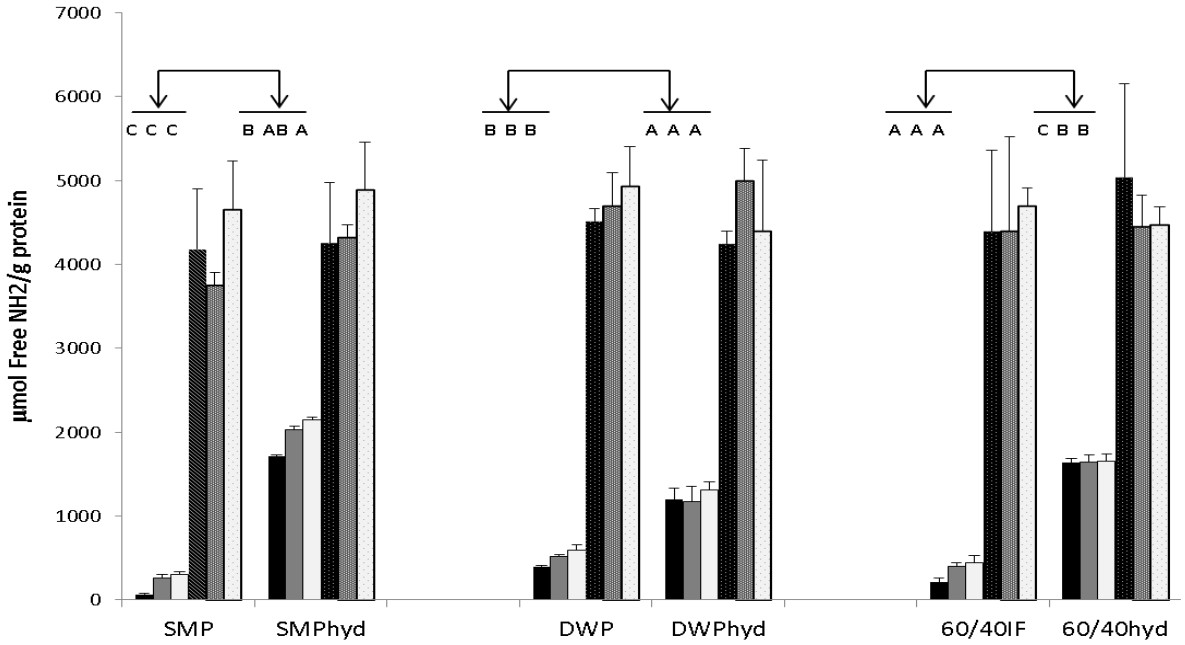

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700 **Fig. 4:** Concentration of free amine groups in µmol per g of protein as determined by OPA
701 assay: Comparison of digesta of SMP, DWP and 60/40IF to SMPhyd, DWPhyd and
702 60/40hyd in the gastric phase at time G0 (▬), G30 (▭), G60 (▭) and intestinal phase at
703 time I15 (▬), I30 (▭) and I60 (▭). Mean values within a column with different
704 uppercase letters (A, B, C) were significantly different ($p < 0.05$), comparison was between
705 gastric time points only.

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